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ORIGINAL ARTICLE

Nucleoside transporters are widely expressed in ovarian carcinoma effusions

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Abstract
Objective Equilibrative and concentrative nucleoside transporters (ENTs and CNTs) mediate the cellular uptake of anticancer nucleosides and sensitivity to such compounds. We studied the expression of ENTs and CNTs in ovarian carcinoma effusions.
Methods ENT1, ENT2, ENT4 and CNT3 expression was analyzed in 66 ovarian carcinoma effusions (61 peritoneal, 5 pleural) from 64 ovarian carcinoma patients by flow cytometry. The majority of patients received platinum-based chemotherapy. Results were analyzed for association with clinicopathologic parameters and survival.
Results With the exception of one ENT2-negative effusion, ENT1, ENT2, ENT4 and CNT3 protein was detected on carcinoma cells in all effusions, with expression observed in 1–95% of tumor cells. Nucleoside transporter expression was comparable between peritoneal and pleural effusions and was unrelated to age, tumor grade, International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumor volume after surgery, previous exposure to chemotherapy and response to chemotherapy at diagnosis ($P > 0.05$). No correlation was found between ENT or CNT expression and overall survival or progression-free survival, although higher ENT2 expression was associated with a trend for longer overall (45 vs. 23 months; $P = 0.055$) and progression-free (17 vs. 5 months; $P = 0.087$) survival.
Conclusion Nucleoside transporters are frequently expressed in ovarian carcinoma effusions, but their expression generally appears to be unrelated to chemoresponse in this cancer in a cohort of patients treated by platinum-based chemotherapy. The role of ENT2 as a prognostic marker in this disease, as well as the role of these molecules in determining chemoresponse in patients treated by nucleoside analogs, merits further research.

Keywords Nucleoside transporters · Ovarian carcinoma · Effusions · Flow cytometry

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Introduction

Ovarian carcinoma (OC) is a highly aggressive disease and the most lethal gynecological malignancy in developed countries. Approximately 21,550 new cases and 14,600 disease-related deaths were estimated in the United States in 2009 [1]. OC involves the peritoneal cavity in the majority of cases and is associated with accumulation of malignant ascites more than any other neoplasm. Pleural fluid is the most frequent manifestation of International Federation of Gynecology and Obstetrics (FIGO) stage IV disease [2]. While a primary ovarian tumor is removed at primary surgery, peritoneal and pleural fluids are often

drained repeatedly in OC patients to relieve symptoms and to confirm the diagnosis of recurrent disease. Malignant cells in effusions are therefore suitable for studies of OC biology not only at diagnosis but also during disease progression.

Equilibrative nucleoside transporters (ENTs) are proteins with a structure of 11-transmembrane α-helices, and transport physiologic nucleosides and chemotherapeutic nucleoside drugs down their concentration gradients. The ENT family is alternatively designated the Solute Carrier 29 (SLC29) family and has four members. The two most studied ENTs are human ENT1 and human ENT2, both first cloned at the end of the 1990s. Human ENT3 and human ENT4 were later identified as a result of completion of the human genome project. The human ENTs are members of a larger group of equilibrative nucleoside and nucleobase transporters found in many eukaryotes [3]. Concentrative nucleoside transporters (CNTs) are sodium-dependent transporters of physiologic nucleosides and chemotherapeutic nucleoside drugs against a concentration gradient. Six different CNTs have been described, but most studies concern CNT1-3 [4].

ENTs and CNTs mediate the cellular uptake of chemotherapeutic nucleoside drugs used to treat malignancies and viral infections, as these hydrophilic molecules require facilitated transport to cross plasma membranes [4]. Anticancer nucleosides interfere with nucleotide metabolism and DNA replication, thereby inhibiting mitosis and promoting apoptotic cell death [5]. ENTs and CNTs are considered important for tumor sensitivity to anticancer nucleosides, but could also promote resistance to the same compounds by mediating salvage of normal nucleosides to overcome toxicity [4].

To clarify the potential value of ENTs and CNTs in OC, either as direct transporters of anticancer nucleosides or as predictive markers for drug sensitivity, studies of their expression in OC cells are needed. Immunohistochemistry performed on biopsy material from 90 primary OC patients has previously revealed ENT1, ENT2 and CNT1 expression in 91, 84 and 67% of tumors, respectively [6]. Ferrandina et al. [7] reported significantly lower ENT1 mRNA expression in a gemcitabine (an anticancer nucleoside drug used in the treatment of recurrent OC)-resistant OC cell line compared to corresponding gemcitabine-sensitive wild-type cells. However, ENT1 and CNT1 expression in 25 OC primary tumors was unrelated to clinical outcome. This could be explained by the small sample size and limited number of events in the study, but may additionally reflect the fact that of the 25 patients studied, only 14 were treated with nucleoside-based salvage therapy after failure of platinum-based therapy, making the group a heterogeneous one.

In the present study, we studied the expression of three ENT and one CNT family members in OC effusions, using antibodies specific for ENT1, ENT2, ENT4 and CNT3. The association between the presence of these proteins and clinicopathologic parameters, including chemoresponse and survival, was studied.

Materials and methods

Patients and material

Effusions and clinical data were obtained from the Department of Gynecologic Oncology at the Norwegian Radium Hospital. Fresh, nonfixed effusions ($n = 39$; 34 peritoneal, 5 pleural) were obtained from 37 patients diagnosed with OC ($n = 33$) or primary peritoneal serous carcinoma ($n = 4$). All will be referred to as OC in this paper because of their similar histogenesis, morphology and treatment. The majority of patients (35/37; 95%) received platinum-based chemotherapy at diagnosis. Twenty-nine effusions were obtained at diagnosis, prior to the administration of chemotherapy, and will be referred to as pre-chemotherapy specimens, whereas 8 effusions were obtained after chemotherapy, henceforth referred to as post-chemotherapy specimens. For two specimens, no information was available regarding previous exposure to chemotherapy. Among the 8 post-chemotherapy effusions, 2 were obtained after neoadjuvant chemotherapy for primary disease, 1 was from a patient who was primarily operated and had disease progression on first-line chemotherapy, and 5 were tapped at disease relapse.

Effusions were submitted for routine diagnostic purposes to the Department of Pathology during 2002–2006 and processed immediately after tapping as previously reported [8]. Diagnoses were established using morphology and immunohistochemistry [9]. Clinicopathologic data for the patients are detailed in Table 1.

ENT and CNT expression was additionally analyzed in an independent series of 27 peritoneal effusions obtained from patients operated for OC at Ulleval University Hospital during 2003–2007. These effusions were all pre-chemotherapy specimens. The majority (23/27; 85%) of patients included in this validation cohort received post-operative platinum-based chemotherapy. Clinicopathologic data for these patients are presented in Table 2.

The Regional Committee for Medical Research Ethics in Norway approved the study of both OC cohorts.

Antibodies and controls

Rabbit polyclonal antibodies against ENT1 (anti-SLC29A1), ENT2 (anti-SLC29A2) and CNT3 (anti-SLC28A3) were all Sigma Prestige Antibodies (Sigma–Aldrich, St. Louis MO), whereas the ENT4 (SLC29A4) mouse monoclonal antibody

Table 1 Clinicopathologic data of the Norwegian Radium Hospital cohort (37 patients)

Parameter	Number of patients
Age	
Range (mean)	42–88 (62)
FIGO stage	
II	1
III	24
IV	12
Grade	
I	4
II	9
III	21
NA[a]	3
Histology	
Serous	31
Non-serous[b]	5
Unknown	1
Residual disease	
≤1 cm	21
>1 cm	12
NA[c]	4
Chemoresponse at diagnosis	
Complete	18
Incomplete[d]	19
Not evaluated[e]	19

[a] *NA* not available; including 1 effusion from an inoperable patient where biopsy was too small for grading and 2 effusions from patients operated in other hospitals, for which the primary tumor could not be accessed for the assessment of grade

[b] Two clear cell carcinomas and three carcinomas of mixed type

[c] Patients with no record

[d] Partial response/stable disease/progression

[e] Including three patients with allergic or adverse reaction

Table 2 Clinicopathologic data of the Ulleval hospital cohort (27 patients)

Parameter	Number of patients
Age	
Range (mean)	45–87 (66)
FIGO stage	
III	18
IV	9
Grade	
II	13
III	12
NA[a]	2
Residual disease	
≤2 cm	6
>2 cm/no surgery	14
NA[a]	7

[a] *NA* not available

was from Abcam (Cambridge, UK). In analysis of several ovarian and breast carcinoma cell lines, the breast carcinoma cell line SKBr-3 was shown to express all 4 proteins (Fig. 1) and was subsequently used as control in analysis of the effusion specimens. SKBr-3 cells were cultured as recently described [8].

Flow cytometry (FCM) immunophenotyping

Four-color FCM was undertaken using the FACSCalibur flow cytometer (BD Biosciences Pharmingen, San Jose, CA). For each specimen, data from at least 500 viable cells were collected. Prior to staining, effusion specimens were processed as previously reported [8]. Briefly, ENT/CNT antibodies and F(ab')2 fragment donkey anti-rabbit or anti-mouse IgG, PE-conjugated (Jackson ImmunoResearch Laboratories, Inc., Suffolk, England), were applied as follows: In the first step, FITC-, PE-, PerCP- and APC-conjugated primary antibodies (isotype mouse IgG1, isotype rabbit IgG (Abcam), mouse Ber-Ep4, mouse CD45 and mouse EpCAM) and non-conjugated ENT/CNT antibodies for surface staining were added to the respective tubes. Cells were incubated in the dark at room temperature for 30 min. After washing twice with 2 ml incubation buffer, PE-conjugated secondary donkey anti-rabbit or anti-mouse antibody was then added to the relevant test tubes, which were incubated for 25 min in the dark, and thereafter washed. FITC-, PE-, PerCP- and APC-conjugated primary antibodies (isotype IgG1, IgG2a, Ber-Ep4, CD45 and EpCAM) and 7-amino actinomycin D (7-AAD) staining solution (BD Biosciences Pharmingen), detailed in Tables 3 and 4, were applied as previously described [8, 10, 11].

Controls/instrument settings: Control of instrument performance and time delay calibration were achieved using FACSComp software version 4.1, Calibrite™ 3 beads and Calibrite™ APC beads (all from BD Biosciences Pharmingen) for four-color FCM setup as previously detailed [8].

Evaluation and scoring of FCM immunophenotyping: Analysis of FCM results was undertaken in a standardized way using the CellQuest™ Software version 3.4 (BD Biosciences Pharmingen) as previously detailed [8]. Quadrant cursors were set using isotypic negative controls and PE-conjugated secondary donkey anti-mouse and donkey anti-rabbit antibodies for the ENT4 and ENT1/ENT2/CNT3 assays, respectively. The percentage of viable carcinoma cells expressing Ber-EP4, EpCAM and each of the 4 nucleoside transporters was scored. Expression in <1% of cells was scored as negative.

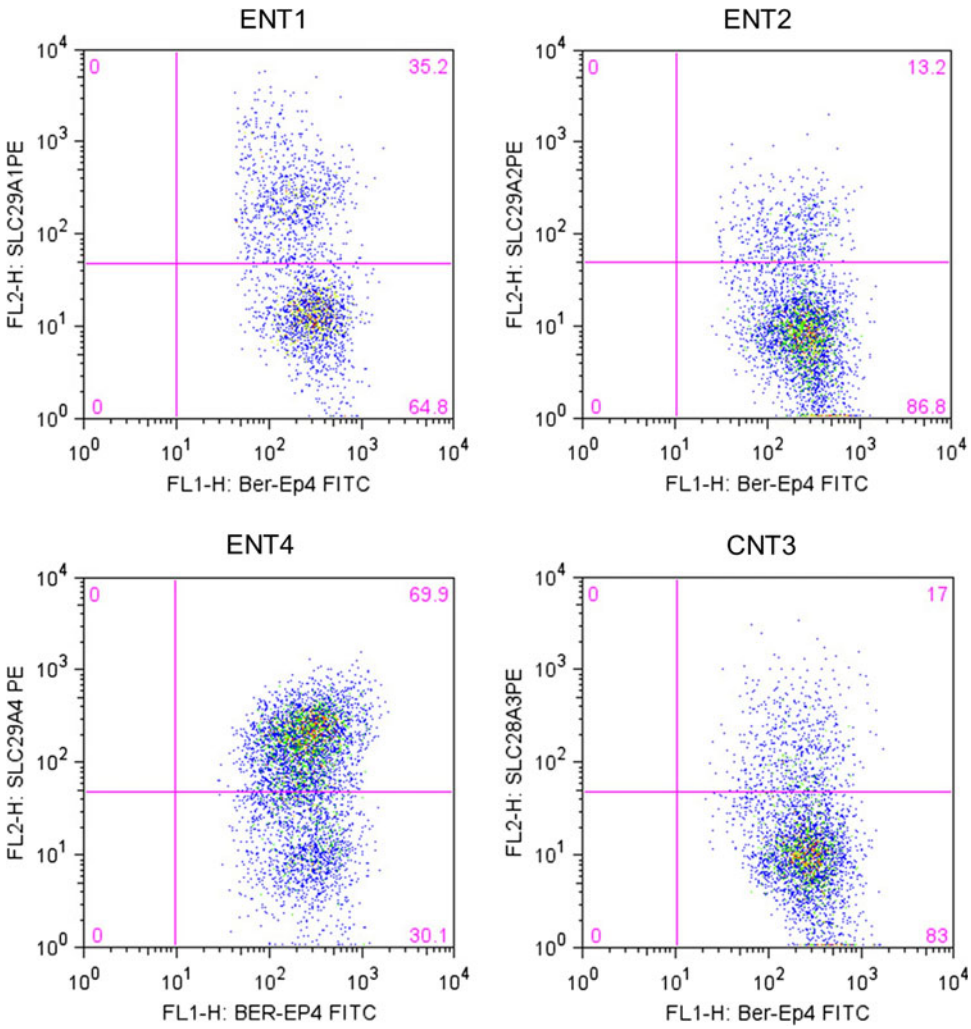

Fig. 1 Flow cytometric analysis of nucleoside transporter expression in SKBr-3 breast carcinoma cells. The breast carcinoma cell line, used as control, expresses ENT1, ENT2, ENT4 and CNT3, with a percentage of positive cells ranging from 13.2 to 69.9%

Statistical analysis

Statistical analysis was performed applying SPSS (Statistical Program of Social Sciences, version 17.0, Chicago, IL). *P* values <0.05 were considered statistically significant. Analysis of the association between ENT/CNT expression in OC cells and clinicopathologic parameters was undertaken using the Mann–Whitney *U* test. For this analysis and for survival analyses, clinicopathologic parameters were categorized as follows: age: ≤ 60 versus >60 years; grade: 1–2 versus 3; effusion site: peritoneal versus pleural; FIGO stage: III versus IV; residual tumor volume after first surgery: ≤ 1 cm versus >1 cm; chemotherapy status: pre- versus post-chemotherapy specimens; response to chemotherapy at diagnosis: complete versus partial response/stable disease/progression. Chemoresponse was assessed based on standard WHO criteria [12].

Progression-free survival (PFS) and overall survival (OS) were calculated from the date of diagnosis to the date of recurrence/death or last follow-up. Univariate survival analyses of PFS and OS were executed using the Kaplan–Meier method and log-rank test. For survival analyses, ENT/CNT expression was grouped as high versus low based on the median value. For patients with more than one effusion analyzed by FCM, only the chronologically first submitted specimen was included in the survival analysis.

Results

Viable OC cells in effusions commonly express nucleoside transporters

Viable OC cells were detected in all effusions using the Ber-EP4 and 7-AAD antibodies. In the Norwegian Radium Hospital series, ENT1, ENT2, ENT4 and CNT3 were detected on carcinoma cells in all 39 effusions, with the exception of one ENT2-negative effusion (Figs. 2, 3). Expression range and median were as follows: ENT1: 1–68%, 23%; ENT2: 0–47%, 11%; ENT4: 3–87%, 21%; and CNT3: 1–77%, 26%.

Table 3 Flow cytometry antibody combinations in the ENT1, ENT2 and CNT3 assay

Tube no.	Test	Primary antibodies for surface staining				Secondary antibody
1	Control of overall background staining	Isotype IgG1 FITC	Isotype rabbit IgG PE	CD45 PerCP/ 7-AAD	Isotype IgG1 APC	
2	Control of background staining of the secondary donkey anti-rabbit antibody in the cancer cell population	Ber-Ep4 FITC		CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-rabbit IgG PE
3	SLC29A1 expression in the cancer cell population	Ber-Ep4 FITC	SLC29A1	CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-rabbit IgG PE
4	SLC29A2 expression in the cancer cell population	Ber-Ep4 FITC	SLC29A2	CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-rabbit IgG PE
5	SLC28A3 expression in the cancer cell population	Ber-Ep4 FITC	SLC28A3	CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-rabbit IgG PE

Table 4 Flow cytometry antibody combinations in the ENT4 assay

Tube no.	Test	Primary antibodies for surface staining				Secondary antibody
1	Control of overall background staining	Isotype IgG1 FITC	Isotype IgG2a PE	CD45 PerCP/ 7-AAD	Isotype IgG1 APC	
2	Control of background staining of the secondary donkey anti-mouse antibody in the cancer cell population	Ber-EP4 FITC		CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-mouse IgG PE
3	SLC29A4 (ENT4) expression in the cancer cell population	Ber-EP4 FITC	ENT4 (SLC29A4)	CD45 PerCP/ 7-AAD	EpCAM APC	Donkey anti-mouse IgG PE

FITC fluorescein isothiocyanate, *PE* phycoerythrin, *PerCP* peridium chlorophyll protein, *APC* allophycocyanin

ENT and CNT expression in effusions is unrelated to clinicopathologic parameters

We analyzed the association between ENT1, ENT2, ENT4 and CNT3 expression in OC cells and clinicopathologic parameters. Expression of the 4 nucleoside transporters was comparable between peritoneal and pleural effusions ($P > 0.05$, data not shown). Their expression was similarly unrelated to patient age, tumor grade, FIGO stage, residual disease volume after surgery and previous exposure to chemotherapy ($P > 0.05$, data not shown).

ENT and CNT expression does not predict response to chemotherapy

All 37 patients received chemotherapy at diagnosis. This consisted of combination of carboplatin and paclitaxel ($n = 30$), carboplatin alone ($n = 3$), carboplatin + paclitaxel + epirubicin ($n = 2$) or other drugs ($n = 2$). Response to chemotherapy at diagnosis could be assessed in 35 cases and was as follows: complete response = 18, partial response = 9, stable disease = 2 and progression = 5. The 3 remaining patients had allergic or adverse reaction.

ENT and CNT expression was unrelated to response to chemotherapy for primary disease ($P > 0.05$, data not shown).

The expression of ENT and CNT transporters in OC effusions does not significantly correlate with survival

The value of ENT1, ENT2, ENT4 and CNT3 in predicting survival was considered. Follow-up ranged from 4 to 156 months (mean = 30 months, median = 20 months). PFS ranged from 0 to 110 months (mean = 11 months, median = 4 months). At the last follow-up, 2 patients were alive without disease, 4 were alive with disease, and 31 were dead of disease. Patients with effusions in which carcinoma cells had higher than median ENT2 expression had longer overall (45 vs. 23 months) and progression-free (17 vs. 5 months) survival compared to those with lower than median levels, although both findings failed to reach significance in univariate survival analysis ($P = 0.055$ and $p = 0.087$ for OS and PFS, respectively). Expression levels of the 3 remaining transporters were unrelated to OS or PFS ($P > 0.7$ for all analyses, data not shown).

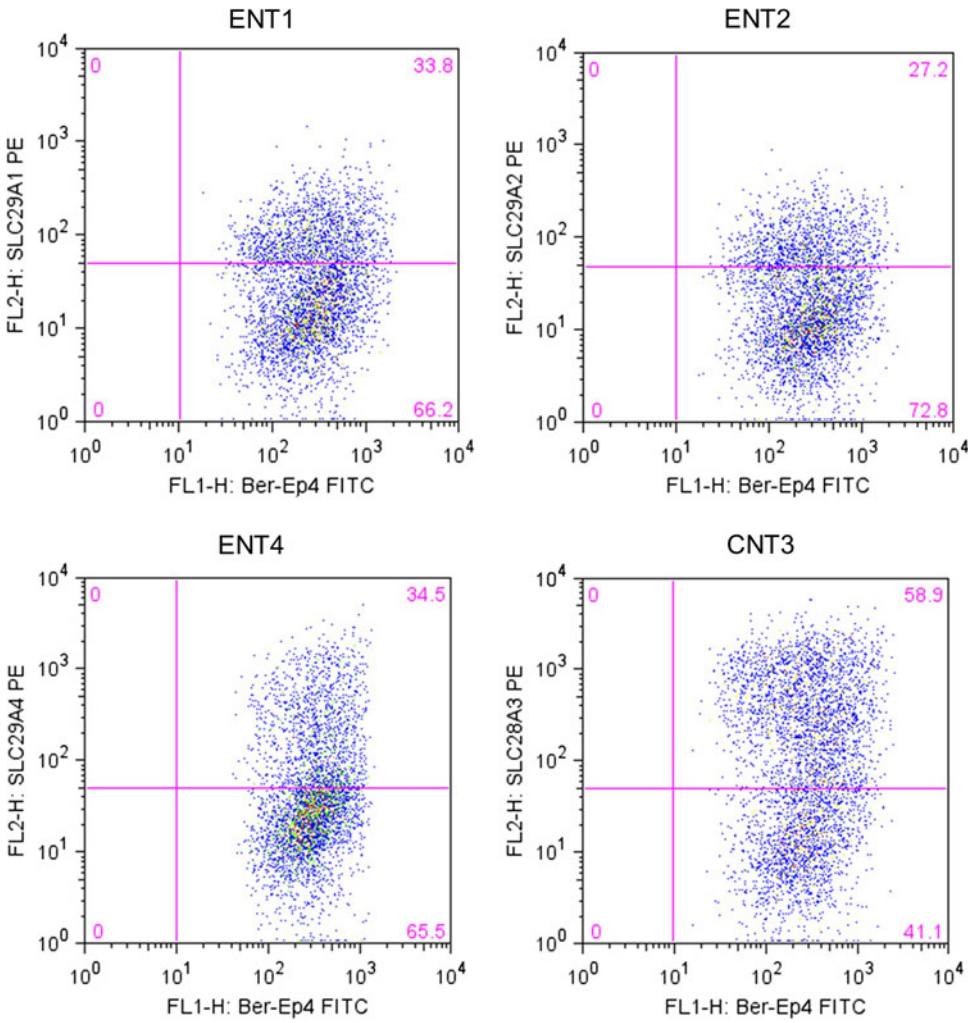

Fig. 2 Flow cytometric analysis of nucleoside transporter expression in ovarian carcinoma cells in effusions. Nucleoside transporter expression in ovarian carcinoma cells in pleural effusion. Tumor cells have high expression of ENT1, ENT2, ENT4 and CNT3, ranging from 27.2 to 58.9%

Validation of the above-described results in an independent OC cohort

To test whether our results could be reproduced, we analyzed ENT1, ENT2, ENT4 and CNT3 expression in 27 effusions obtained from an independent cohort of 27 OC patients. Comparable to the findings described above, tumor cells in all 27 effusions expressed ENT1 (expression range 3–95%; median = 46%), ENT2 (expression range 1–81%; median = 17%), ENT4 (expression range 3–85%; median = 34%) and CNT3 (expression range 3–94%; median = 36%). As in the main cohort, ENT1, ENT2, ENT4 and CNT3 expression was independent of age, histological grade and FIGO stage ($P > 0.05$, data not shown).

For the 27 patients in the validation cohort, residual tumor volume after chemotherapy was grouped as ≤2 cm versus >2 cm/not undergone primary surgery. ENT1, ENT2, ENT4 and CNT3 expression was unrelated to residual tumor volume after surgery ($P > 0.05$), in agreement with the findings described above.

Data in response to chemotherapy were not available for the validation cohort. However, we looked for a potential association between ENT1, ENT2, ENT4 and CNT3 expression and CA125 concentration in serum obtained at diagnosis prior to surgery. CA125 concentration was comparable for patients with tumor cell expression below and above the median for all nucleoside transporters ($P > 0.05$, data not shown).

Despite the limited number of patients, the value of ENT and CNT proteins in predicting survival was also studied in this cohort. Follow-up ranged from 1 to 82 months (mean = 24 months, median = 22 months). PFS ranged from 0 to 82 months (mean = 9 months, median = 3 months). At the last follow-up, 1 patient was alive without disease, 1 patient was alive with disease, and 25 patients were dead of disease. No significant association was found between the expression of ENT1, ENT2, ENT4 and CNT3

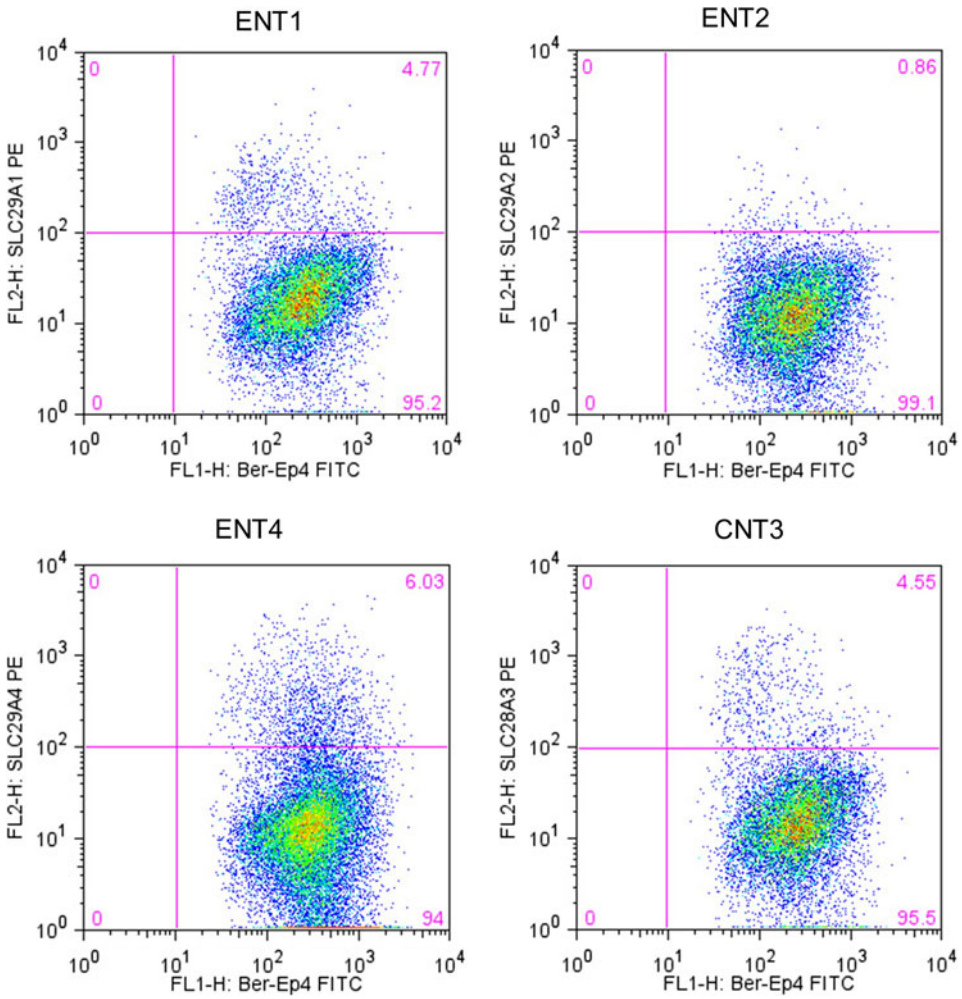

Fig. 3 Flow cytometric analysis of nucleoside transporter expression in ovarian carcinoma cells in effusions. Ovarian carcinoma cells in a peritoneal effusion, with more limited expression of the studied proteins, ranging from 0.86 to 6.03%

and OS or PFS in this cohort ($P = 0.1$–0.89, data not shown).

Discussion

Initial treatment of advanced-stage OC is radical surgical debulking followed by adjuvant chemotherapy. Intravenous administration of a platinum compound in combination with paclitaxel is currently first-line therapy. This regimen prolongs both PFS and OS, but most patients will eventually relapse and require further treatment [13, 14]. Among the most investigated drugs in treatment of recurrent OC is the anticancer nucleoside gemcitabine [15–17]. To exert their intracellular cytotoxic effects, anticancer nucleosides depend upon transport across the plasma membrane by nucleoside transporters. We therefore hypothesized that OC cell ENT and CNT expression could be important for sensitivity to such compounds and also for patient outcome. In the present study, the expression of ENT1, ENT2, ENT4 and CNT3 was studied in two independent series consisting of a total of 66 OC effusions. To the best of our knowledge, this is the first study of nucleoside transporter expression in OC effusion cells.

FCM, used to detect nucleoside transporter expression in the present study, is a quantitative method, which has high sensitivity and reproducibility. It allows the study of multiple molecules simultaneously and enabled us to differentiate between carcinoma cells, leukocytes and mesothelial cells by employing a panel of markers, including Ber-EP4 and CD45 [18]. 7-AAD intercalates into double-stranded nucleic acids and is taken up only by cells with damaged cell membranes. The addition of 7-AAD made it possible to exclude dying and dead cells from the analysis, as these often express genes or proteins in a pattern that is unrepresentative for viable cells.

The 4 transporters were essentially universally expressed in specimens from both cohorts. ENT1 and CNT1 mRNA expression was previously observed in primary OC using real-time quantitative-PCR [7], whereas Pennycooke et al. found that CNT1 and CNT2 mRNAs were expressed in both normal ovarian tissue and OC cells, with higher

expression seen in the latter [19]. In an additional study, ENT1, ENT2 and CNT1 protein expression was found in ovarian, cervix and endometrial carcinomas using immunohistochemistry [6]. Our data are in agreement with the above reports with respect to ENT1 and ENT2. CNT1 and CNT2 expression was not analyzed in the present study, as we failed to find antibodies detecting these proteins in human cells that perform well in the FCM assay.

Heterogeneous expression of ENT1, ENT2, ENT4 and CNT3 was seen in OC effusions, strongly supporting the existence of biological variability. This allows for stratification of patients according to the abundance of protein expression, in order to attempt a correlation with clinicopathologic parameters or disease outcome.

Nucleoside transporter expression was unrelated to any of the clinicopathological parameters in both cohorts analyzed in the present study. However, in our OC effusion series from the Norwegian Radium Hospital, higher ENT2 expression was associated with a considerably longer OS and PFS, though not significantly so. The association between higher ENT2 levels and longer survival is in agreement with a previous report, in which ENT1 immunostaining in tumor cells correlated with prolonged survival after gemcitabine therapy for pancreatic adenocarcinoma patients, compared to patients whose tumors did not express ENT1 [20, 21].

The failure of the survival analysis to reach significance in our study may owe to the relative small number of patients in this cohort and, though not reproduced in the small cohort from Ulleval Hospital, merits further research of larger patient material.

We conclude that among ENT1, ENT2, ENT4 and CNT3 are frequently expressed by OC cells in effusions. Of these 4 transporters, only ENT2 may have prognostic value, while none of the studied molecules was significantly associated with chemotherapy response. It is nevertheless important to note that the majority of effusions were tapped from patients treated mainly with carboplatin in combination with cisplatin. Only a minority of the included patients received gemcitabine therapy following disease relapse. Thus, the predictive value of ENT and CNT family members may emerge from analysis of a different OC cohort with more patients treated with anticancer nucleoside drugs. Further studies are needed to explore the expression and clinical role of ENTs and CNTs, especially ENT2, in gemcitabine-treated OC patients.

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Conflict of interest None.

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